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Distribution and Metabolism of Tritiated Yohimbine in Mice [1]

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Abstract—Studies of the distribution of tritiated yohimbine in mice revealed that this drug rapidly penetrated the blood-brain barrier and accounted for 95 % of the total radioactivity present in the brain within 10 min. The half life of yohimbine in the brain was estimated to be 3 hr. There was no marked differential distribution of total tritiated material in the different topographical regions of the brain over the time course studied. A study of the subcellular distribution revealed that labeled yohimbine was highest in the synaptosomal subfraction. The distribution of both total radioactivity and labeled yohimbine in other organs over a 24 hr time period is also reported. Radiochromatographic analysis of tissue extracts revealed the presence in urine and in other tissues of at least two radioactive metabolites in addition to yohimbine itself. The significance of these findings is discussed particularly with respect to the well known adrenergic blocking activity of yohimbine, and also in relation to the distribution and duration patterns of the corresponding Rauwolfia alkaloid, reserpine.

Introduction

Yohimbine, an indole alkaloid, has long been known to possess adrenergic blocking proporties (1-3) and recently its effect on tryptophan pyrrolase has been demonstrated (4). Other pharmacological actions of yohimbine include its local anesthetic, vasodilator, antidiuretic, and aphrodisiac properties. Despite its pharmacological activities, yohimbine has had relatively little use as a therapeutic agent (5). However, the possibility of the clinical use of yohimbine as an

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autonomic test drug was recently suggested (6-7) and some evidence of therapeutic effect in endogenous depression was noted. It was observed that in man a test dose of yohimbine produces marked autonomic and psychic effects similar to those in anxiety states. It was further found that yohimbine potentiates the antidepressant activity of imipramine in conscious man and animals (6, 8, 9, 24). Yohimbine, in combination with methyl testosterone and nux vomica, has also been used for the treatment of impotence in man (10-11). However, in spite of its increasing use both clinically and experimentally, the distribution and metabolism in animals and man has not been studied. Furthermore, the similarities in structure between yohimbine and reserpine and their opposite behavorial effects also attracts our attention.

Methods

Twenty-four adult male Swiss-Webster mice of about 25 g in weight were injected i.p. with yohimbine-methyl-^{3}H (specific activity : 5 mc in 2.36 mg, lot number 184-106) (1). The purity of the labeled drug was examined using the techniques of radiochromatographic scanning described below, and only one peak corresponding to yohimbine was obtained. The injection solution (0.25 ml) to each animal contained 250 μc of yohimbine-methyl-^{3}H diluted with yohimbine hydrochloride solution to a final dose of 10 mg/kg. Immediately after injection the animals were placed in metabolic cages to facilitate collection of urine. The urine samples were collected in the intervals prior to sacrifice, with the first collection period being at 3 hr. The animals were killed at 10, 30, 45 min and 1.5, 3, 6, 12, and 24 hr after administration. Blood samples were collected immediately following decapitation. Different parts of the CNS, including the spinal cord, brain stem (medulla and pons), cerebellum, hypothalamus, diencephalon and the cerebral cortex were dissected for examination. Tissue samples from the heart, spleen, liver, kidney (mixed with cortex and medulla), adrenal glands, small intestine (freed of intestinal contents) and rectus abdominis muscle were blotted with filter paper and weighed on a torsion balance. Each experiment for a given time interval was run in duplicate from a pooled tissue sample of 2 mice and the organs assayed individually.

Extraction and *Analysis*. Each tissue specimen was finely minced and then extracted by shaking with acetone (volume approximately 20 times that of the tissue). The procedure was then repeated and the tissue allowed to remain in the acetone solution over a 48-hr period. Both extracts for a given tissue were combined. In the case of urine, a mixed solvent of acetone and benzene (1 : 4) was used. In previous control runs it was determined that this procedure did produce near complete extraction of radioactive yohimbine from tissues. Thus, 2 animals were injected i.p. with yohimbine solutions indentical to that previously

(1) Obtained from New England Nuclear Corporation.

described. Different organs from each animal were isolated and equally divided between 2 sets of test tubes. Aliquots containing 0.05 μc yohimbine-^{3}H were then added to one set of tubes. Both sets of tubes were then extracted as described above.

The recovery of the yohimbine-^{3}added externally to the selected tubes was approximately 90 %. Furthermore, when the tissue residues obtained from the above procedure were dissolved in 1 ml of hyamine reagent (1 M in methanol), (1) the amount of radioactivity obtained from each residue was less than 7 % of that contained in the acetone extract. The effectiveness of the above acetone extraction was thus established.

Aliquots of 100 μl taken from the final extracts were mixed with a scintillation counting fluid, (2) and the total tritium content was determined in a Beckman Model LS200B Liquid Scintillation Counter. All sample counts were corrected for acetone quenching by the addition of internal standard to each counting sample. The remainder of the acetone extract from each specimen, or pooled specimens (as in the case of the CNS), was concentrated by evaporation with a stream of N_2 gas, and the concentrate was subjected to thin layer chromatography to determine the presence of yohimbine and metabolites. Aliquots of the concentrate were spotted on instant TLC paper (3) and developed in an ascending chromatographic system containing hexane, chloroform, and diethylamine, in a ratio of 5 : 4 : 1. The developed strips were dried in an oven at 30 °C, examined under ultraviolet light for the presence of fluorescent spots, and scanned by a Packard Model 7200 Radiochromatographic Scanner with Packard Model 385 Recording Rate Meter, to determine the location and relative quantities of radioactive materials. The areas under the peaks observed in the radiochromatograms were determined and used to calculate the percentage of yohimbine present in each organ at various time intervals. Some of the spots on the chromatograms were also extracted with acetone and the tritium content was counted.

Subcellular Fractionation. One and a half hr following i.p. injection, the brains from 6 mice were homogenized individually in 0.32 M sucrose. Differential centrifugation was carried out as described by Whittaker (12) into 'nuclear' (P_1), 'crude mitochondrial' (P_2), 'microsomal' (P_3), and 'cytoplasmic' (S) subcellular fractions. The P_2 fraction was further separated into the subfractions : 'myelin-membrane fragment' (A), 'synaptosome' (B), and 'mitochondria' (C), by centrifugation of 53, 500 rpm for 120 min on a density gradient consisting of equal volume of 0.8M and 1.2M sucrose. Tissue samples from heart, liver, kidney, spleen and intestine were also subjected to differential centrifugation and fractions were isolated in the same manner as was used to obtain the P_1, P_2 and P_3 fractions described above. The total tritium content and percent tritiated yohimbine for each fraction obtained were determined as previously described. The

(1) Obtained from Packard Company.
(2) Obtained from Nuclear Chicago Company.
(3) Obtained from Gelman Instrument Company.

percentage of total nitrogen in the various subcellular fractions was measured by microdetermination of Kjeldahl (13) as an expression of total protein content in different fractions.

Results

The distribution in various organs of total radioactive materials at different time intervals after a single dose of tritiated yohimbine is summarized in Table I and Fig. 1. Tritium accumulated rapidly in the brain (within 10 min), reached a peak at 30 min, and then gradually declined. It is noteworthy that at the maximum concentration at 30 min, the total tritium content present in the brain was 0.95 % of the injected labeled yohimbine, and only 0.65 % was found to be the

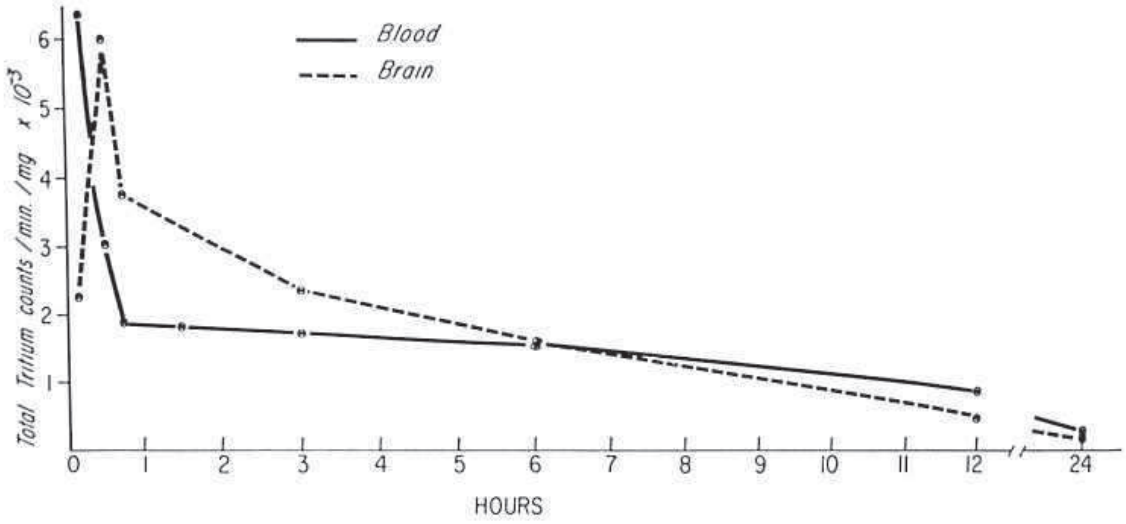

FIG. 1

^{3}H content of blood and brain as a function of time after i.p. injection of 250 μc yohimbine-^{3}H.

parent compound. The different parts of the brain examined showed the same variation of labeled material with time, and no one area showed a markedly different concentration than any other. In the blood, total tritium content reached a maximum within 10 min, decreased rapidly during the next 45 min, followed by a slower, steady decline. In the peripheral organs uptake and release of tritiated material reveal some differences among the various organs. The rate of accumulation of tritium in the spleen, adrenal glands, intestine, liver and muscle was greatest at 10 min after injection, whereas in the heart it reached a peak at 30 min. It is probable that the high radioactivity present in the organs within the peritoneal cavity only at the 10 min interval is due to some contamination from i.p. administration.

In general, the total tritium content in tissue showed a progressive decrease

TABLE I

Total radioactivity at different time intervals after yohimbine-^3H administration in mice expressed as count/min per mg tissue wet weight

Tissue	10 min	30 min	45 min	1.5 hr	3 hr	6 hr	12 hr	24 hr
Whole Brain (1)	2,275							
Cerebral Cortex	—	6,190	4,620	3,850	2,890	2,000	580	270
Cerebellum	—	5,760	3,470	2,980	1,980	1,380	520	180
Hypothalamus	—	5,460	3,595	3,240	2,320	1,590	450	110
Diencephalon	—	6,330	3,610	3,610	2,650	1,870	500	210
Brain Stem	—	5,750	3,560	2,940	2,160	1,390	470	160
Spinal Cord	—	3,670	2,440	2,890	2,780	1,680	400	10
Blood	6,180	3,050	1,990	1,860	1,750	1,590	920	310
Heart	6,520	8,890	5,100	6,310	4,000	4,530	1,840	470
Spleen	19,250	14,020	15,830	10,320	9,710	8,890	6,190	670
Kidney	21,500	23,520	17,230	17,260	17,470	18,550	6,360	21,70
Adrenals	25,900	15,700	9,810	—	6,410	5,140	2,500	360
Liver	29,200	28,810	22,620	21,840	11,560	13,050	4,850	1,350
Intestine	26,400	16,170	13,600	14,550	13,160	7,190	4,670	1,030
Muscle	34,900	7,210	3,930	2,510	3,050	3,300	1,080	150

(1) Spinal cord not included. Each value represents a pooled tissue sample from 2 mice. Whole brain only was analyzed at 10 min.

with time, although there were some differences among tussues. From 30 to 90 min after administration the liver had the highest radioactivity, followed by the kidney, intestine, and the adrenal glands. The heart, skeletal muscle and blood had the lowest. From 3 hr onward the kidney had the highest counts, and after 6 hr both the kidney and liver retained markedly greater amounts than the other organs. The heart and muscle lost more of their total radioactivity at 12 hr than the other organs. At 24 hr the largest amounts of the remaining total radioactivity were found, in dereasing order, in the kidney, liver, and intestine. Total tritium content excreted in the urine over 24 hr was 1.2×10^8 counts/min, a recovery of approximately 22 % of the injected labeled material.

The amounts of labeled yohimbine present in the brain, blood, heart, spleen, liver and urine at different time intervals were examined using the technique of radiochromatographic scanning (Table II). Within 10 min after administration yohimbine was present in the brain and at a value of 95 % of the total radioactivity in that organ. The percentage of radioactive material as yohimbine present in the CNS at the time intervals of 45 min, 90 min, and 3 hr was 67 %, 74 % and 51 % respectively. It then dropped to 17 % at 6 hr and none could be detected from 12 hr onward. The percentage of labeled yohimbine decreased in the blood during the initial periods, and then remained relatively constant over the 1.5 to 12 hr interval (47-38 %), possibly reflecting its steady release from tissue. In general, all the other organs showed a progressive decrease in the levels of radioactive yohimbine. The liver showed a maximum value (76 %) of yohimbine-^{3}H at 1.5 hr and was the only organ that still retained a percentage of yohimbine (6 %) after 24 hr. No yohimbine-^{3}H could be detected in the intestine at 10 min, although levels of 63 % and 73 % respectively were present at the 45 and 90 min intervals. However, the drug was relatively absent in the intestine from the 3 hr interval onward.

Fig. 2 illustrates the separation by thin layer chromatography of tritiated yohimbine from its labeled metabolites in extracts of urine, spleen and liver at selected time intervals, the areas under the curves representing the relative amounts of radioactivity present. Positive identification of yohimbine and its metabolites requires the presence of a clearly defined fluorescent spot at the face of a specific radioactive peak, where both the spot and the peak correspond to a known reference standard. In all tissues examined the radioactivity of labeled yohimbine was associated with a discrete fluorescent spot which migrated with an average $R_f \pm$ S.E. value of 0.91 $\pm$ 0.05, and yohimbine-^{3}H always appeared as the last peak before the solvent front. Other peaks (not yohimbine) with R_f values ranging from 0.54-0.84 were present, some with and some without fluorescense. In addition to these components, several extracts from each organ gave radioactive peaks of unknown identity at the origin of the chromatogram.

It should be noted, however, that not all extracts for a given tissue revealed the same number of components for every time interval examined. The unequivocal interpretation of the radiochromatograms was rendered difficult by the

TABLE II

Percentage of total tritiated material as yohimbine-^3H in mouse tissues at different time intervals

Tissue	10 min	45 min	1.5 hr	3 hr	6 hr	12 hr	24 hr
Whole Brain	95						
CNS	—	67	74	51	17	Trace	Trace
Heart	95	54	39	43	33	Trace	Trace
Liver	62	59	76	28	38	11	6
Spleen	95	78	69	56	45	29	Trace
Blood	95	66	47	38	33	38	Trace
Intestine	Trace	63	73	Trace	9	Trace	Trace
Urine (1)	—	—	—	54	33	18	37

(1) No urine obtained in the 10-min to 1.5-hr interval. Each value represents data obtained from pooled tissue samples of 2 mice. Trace amount represents less than 1 % of total radioactivity.

presence in many extracts of large amounts of non-specific background fluorescence. Such interfering material is a likely cause of the observed variations in R_f values between possible identical metabolites from the various organ extracts.

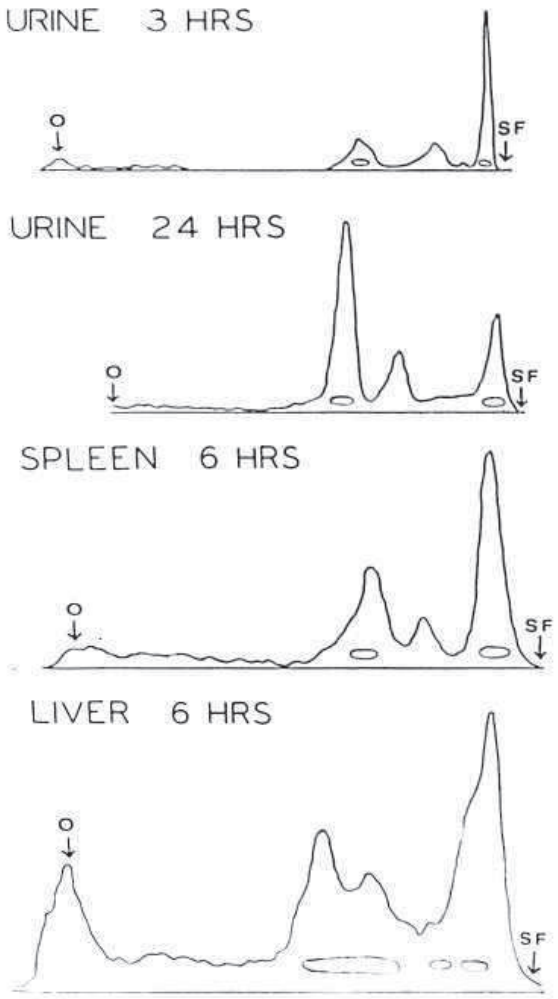

FIG. 2

Thin layer chromatograms of acetone-benzene (1 : 4) and acetone extracts of urine, spleen and liver at different time intervals showing separation of yohimbine-^{3}H and labeled metabolites. Solvent system: hexane, chloroform, diethylamine (5 : 4 : 1). O: origin; SF: solvent front. Mark at base of peak indicates fluorescent spot. Yohimbine appears as the last peak before the solvent front.

In the urine extracts at least 3 distinct peaks were found. A comparison (Table II) of the composition of the extracts at the 2 time intervals (Fig.2) reveals that at 24 hr significantly less yohimbine (37 %) was present than at 3 hr (54 %), the difference being due to the increased amounts of metabolites being excreted at the later time period. Only one of the two observed metabolites showed a significantly greater rise at the 24 hr period.

In order to determine the subcellular distribution of radioactive material in

TABLE III

Distribution of radioactive material in subcellular fractions of various mouse organs expressed as: per cent of total recovered tritiated material per organ: Col. (1) and as per cent total recovered yohimbine-^3H per organ: Col. (2)
Mean ± S.E. (6 Mice)

Fraction	CNS		Heart		Liver		Kidney		Spleen		Intestine	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
P_1 Myelin, nuclei & cell fragments (1000 *g* × 11 min)	19.7 ± 1.39	21.5 ± 1.32	20.6 ± 1.1	18.6 ± 3.3	23 ± 1.3	25.4 ± 1.5	21.4 ± 0.9	21.7 ± 1.9	24 ± 1.4	23.3 ± 1.1	31 ± 2.3	32.3 ± 2.4
P_2 (1) Myelin, membrane fragments, mitochondria, synaptomoses (17,000 *g* × 60 min)	55.5 ± 2.1	53 ± 3.5	41.8 ± 1.8	45.3 ± 2.3	40.2 ± 0.9	43.7 ± 1.7	43.7 ± 1.48	46 ± 2.6	40.3 ± 1.0	41 ± 2.7	41.7 ± 1.6	41.7 ± 1.2
P_3 Microsomes (100,000 *g* × 60 min)	8.3 ± 0.8	6.7 ± 1.3	17.5 ± 0.9	15.2 ± 1.7	10.7 ± 1.0	5.7 ± 0.5	10 ± 0.6	10.2 ± 0.8	12.5 ± 1.2	12.5 ± 1.8	10.0 ± 1.1	8 ± 1.1
S_3 Soluble cytoplasm	17.7 ± 0.8	18.3 ± 2.3	20.2 ± 1.1	19.2 ± 2.2	26.7 ± 1.2	25.8 ± 2.1	25.8 ± 1.6	22.8 ± 2.8	24.3 ± 1.4	24.3 ± 2.4	18.3 ± 2.7	21 ± 2.3

(1) The sub-fractional distribution in the CNS of the radioactive material reported for the P_2 fraction was as follows:

Sub-Fraction	% radioactive material per P_2 fraction Mean ± S.E. (6)	% yohimbine-^3H per P_2 fraction Mean ± S.E. (6)
A. Myelin & membrane fragments	30.7 ± 1.6	29.5 ± 1.54
B. Synaptosomes	49.5 ± 2.5	50.3 ± 2.1
C. Mitochondria	20.8 ± 1.5	19.7 ± 1.73

the various organs, the procedure described by Whittaker (12) for the subcellular fractionation of tissue was employed. In the case of the CNS, the P_1, P_2, P_3 and S fractions were found to contain 32 %, 40 %, 10 % and 19 % nitrogen respectively. An examination of the result (Table III) reveals that the highest relative proportion of both total radioactivity and yohimbine-^{3}H was located in the P_2 fraction (myelin, membrane fragments, mitochrondia, synaptosomes). In the CNS and heart the content of total labeled material and yohimbine-^{3}H in the P_2 fraction was at least twice that observed for any other fraction. When the P_2 fraction obtained from the CNS was further subfractionated, 50.3 $\pm$ 2.1 % and 29.5 $\pm$ 1.6 % of the yohimbine-^{3}H present in the whole fraction was found in the synaptosome and myelin-membrane fragment layers respectively.

Discussion

Yohimbine was readily absorbed, widely distributed and retained in tissue for some time before being metabolized and excreted. Thus, at both the 10 min and 45 min periods between 54 %-95 % of the total radioactive material present in the CNS, heart, liver and spleen was accounted for by yohimbine-^{3}H. In particular, yohimbine penetrates the blood-brain barrier rapidly and a substantial quantity (17 %) is retained in the CNS 6 hr after administration. The half-life of yohimbine in the brain was estimated to be approximately 3 hr. There was no significant difference in total radioactivity distribution in various topographical regions of the brain over the entire time course. However, this was in sharp contrast to the subcellular distribution of the drug, where the data reveal that the concentration of labeled yohimbine was highest in the subcellular P_2 fraction. With further subfractionation in the CNS, the highest levels were in the synaptosomal subfraction, followed by the myelin-membrane fragment and the mitochondrial subfractions.

The procedure employed for the subcellular fractionation was basically that described by Whittaker (12). While this methid was originally applied to rabbit and guinea pig brain, the essential features of it have been successfully extended to the study of mouse brain (14, 15). Enzyme markers and N_2 determinations were carried out as indirect evidence of their biochemical characteristics of various subfractions. The results were in agreement with those reported by Whittaker (12). Although the fractionation procedure was also applied in the current study to tissues from peripheral organs, there is no morphological evidence that these fractions contain exclusively the subcellular particles under investigation.

The preferential affinity of yohimbine for synaptosomes and membrane fragments may indicate that the site of action of this drug is in the nerve terminals. This hypothesis is in accord with, and might contribute to the explanation of the actions of yohimbine as an alpha-adrenergic blocker (1-3, 8), and as a CNS stimulant in man (6-7) and in dogs (8, 16, 17). However, yohimbine adminis-

tered in the dose employed in the present study produced sedation in mice, an effect also observed by Quinton (9). This worker reported a 27 % depletion of brain norepinephrine, and this effect could be in accord with the synaptosomal concentration of yohimbine.

Recently we reported that yohimbine administered chronically over a period of 3 weeks did not lower the levels of serotonin or norepinephrine, but a 50 % increase in the turnover rate of serotonin in the hypothalamus was observed (18). This finding was also considered as in agreement with the proposal that the site of action of the drug is the nerve terminals. As already noted, yohimbine enters the brain readily and remains in this organ several hours after the disappearance of the sedative effects. However, there appears to be a direct relationship between blood levels of the drug and its behavioral effects in dogs and in man (unpublished observation). Further research is currently in progress in our laboratories to associate the observed species differences with specific physiological effects.

It is of particular interest to note that some of the results with yohimbine are in sharp contrast to those obtained with reserpine, an indolic analog of yohimbine, both of which occur naturally in Rauwolfia Serpentia. Reserpine is preferentially distributed in different brain areas such as the hypothalamus, area postrema and subependymal area of the ventricles, as revealed by autoradiographic and tracer studies (19, 20). Furthermore, Sheppard *et al* (21) showed that while most of the reserpine could be found in the particulate matter of the cell, there was no preferential accumulation in any one subcellular fraction. Preliminary findings in our laboratory also revealed that the subcellular distribution of tritiated reserpine was non-specific. Reserpine produces marked CNS depression in all species, an effect related to the depletion of biogenic amines. No direct relationship between tissue levels and behavioral effects seems to exist (22, 23).

The metabolic breakdown of yohimbine was also found to be very rapid as evidenced by the fact that 10 min after i.p. administration no yohimbine but large amounts of metabolites including yohimbinic acid were found in the intestine, while in the liver and kidney 40 % and 60 % of the total tritiated material was in the form of metabolites. It is likely that the major pathway of yohimbine is by hydrolysis of the ester linkage by non-specific esterases present in the intestine. The reappearance in the intestine of yohimbine at 45 min (63 %) and 90 min (73 %) probably reflects the release of the drug from other tissues, whereas its relative absence thereafter reflects the observed presence during this period of drug metabolites. The liver had the highest count of total radioactivity at 30 min and at 90 min, and was the only organ that still retained yohimbine (6 %) at the 24 hr period. Both these observations are in accord with the importance of the liver in the drug detoxification process. Further identification of the yohimbine metabolites obtained from animals and man is currently under investigation.

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